Antitumour and Cytogenetic Effects of Modified Steroidal Derivatives of Propenoic Acid: *In Vivo*/*In Vitro* Studies

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Abstract. *Background: Modified steroidal derivatives (PK11-PK14) of p-bis(2-chloroethyl)aminophenyl propenate (PK15) were used to study their antitumour activity on Lewis lung carcinoma (LLC) and their effect on sister chromatid exchanges (SCEs) and human lymphocyte proliferation kinetics. Materials and Methods: LLC was tested in this study. C57BL mice were used for in vivo chemotherapy evaluation and the antitumour activity was assessed. Lymphocyte cultures were used to study the genotoxic effect in vitro. Results: PK15 and PK11 were the most effective against LLC, causing significant inhibition of tumour growth. PK11 and PK15 induced significant increase in SCE rates. A correlation was observed between the cytogenetic effect and the antitumour effectiveness. Conclusion: The order of the antitumour effectiveness of PK11-PK15 resembled the order of the cytogenetic damage induced by the same compounds in vitro.*

A major aspect of a new drug development programme is the investigation of compounds related to active antineoplastic agents which are synthesised by modification of prototypes. Among the various groups of compounds with antineoplastic activity, alkylating agents, such as chlorambucil and its related compounds CAPP (PK15, Figure 1), in connection with modified steroids are of importance (1, 2). Steroidal derivatives have been used as carriers of cytotoxic agents because they reduce systemic activity and improve the specificity of cancer therapy. The modified steroids act as a biological platform for transporting the alkylating agent specifically to the tumour site (2, 3).

In our experiments, CAPP and the modified steroidal derivative of CAPP (PK11) produced satisfactory inhibition in the growth of the primary Lewis lung carcinoma (LLC) tumour in an animal tumour model). Sister chromatid exchanges (SCEs), on the other hand, have been proposed as a possible *in vitro* or *in vivo* method of evaluating chemotherapy (4). In addition, studies have shown that the determination of proliferation rates in lymphocyte cultures should be a useful and sensitive indicator of the cytostatic activity of chemotherapeutic agents in humans (2, 4). In the present study the effectiveness of these compounds on SCE induction and on the depression of the proliferation rate index (PRI) was tested *in vitro* and positively correlated with the *in vivo* LLC tumour response to these agents.

Materials and Methods

Tumour implantation. LLC tumour was obtained from the Division of Cancer Treatment and Diagnosis, NCI, Frederick, MD, USA and from Tom Corlett of the Southern Research Institute, 431 Aviation Way, Frederick, MD 21701, USA (www.southernresearch.org). C57BL mice of both sexes were used for chemotherapeutic evaluation. The tumour was implanted in the axillary region with puncture in the inguinal region. The sacrificed donor animal was pinned to a dissecting board, with the dorsal surface up. The tumour was transferred to a sterile petri dish placed over ice and debrided of any necrotic material. The tumour was cut into cubes, sized approximately 2×2×2 mm. Using forceps, the tumour fragments were placed into the bevel end of the 3-gauge trocar. The area of the recipient animal was swabbed with 70% ethanol and the tumour fragment was inoculated subcutaneously. The recipient animals were allowed to rest for 24 h and were subsequently divided into the indicated number of groups which received intraperitoneal treatment.

Estimation of acute toxicity. BDF1 mice were used for calculating lethal doses LD_{50} and LD_{10} in groups of 10. They were injected with a single intraperitoneal injection of various doses, ranging from 100 to 800 mg/kg. The mice were observed for 30 days and the LD_{50} and LD_{10} values were estimated graphically, where the

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Key Words: Azasteroids, experimental chemotherapy, human lymphocytes, Lewis lung carcinoma (LLC), sister chromatid exchange (SCE), proliferation rate index (PRI).

percentage of deaths due to the toxicity of each dose was shown in the ordinate, while the administered doses were indicated on the abscissa. For chemotherapy testing, the highest dose used for a single treatment was LD_{10} (Table I). Therefore, the drugs in the antitumour evaluation were compared at equitoxic doses (Tables I, II and Figure 2).

Evaluation of antitumour activity. Standard propagation methods were used for testing the cytostatic effect of the compounds PK11-PK15 (5, 6). The antitumour activity against LLC was assessed from the inhibition of tumour growth (ITG) by volume in cubic centimeters. Tumour size (cm^3) was measured twice per week with a microvernier using the formula (a×b×c)/2 (where a: length, b: width and c: depth at the site of inoculation). The mortality of the animals was monitored daily. The surviving animals were sacrificed on the 60-90th day from the time of inoculation.

Treatment. Experimental groups of 6 mice for each drug tested and a control group of 8 mice were used. Experiments were initiated by implanting mice with the tumour cells. Mice were treated by intraperitoneal (*i. p.*) bolus injection on day 1 after tumour implantation, using the LD_{10} doses of all compounds tested.

In vitro SCE assay. For SCE demonstration, 4 μg/ml of 5-bromodeoxyuridine (BrdUrd) and solutions of the compounds at four concentrations (0.2, 0.6, 2 and 6 μM) were added at the beginning of culture life. The cultures were incubated for 80 h at 37˚C. Lymphocyte cultures were set up by adding 4 drops of heparinised whole blood from normal subjects to 4 ml of RPMI-1640 cell culture medium. The individuals who donated their blood were healthy (18-20 years old), were not taking any medication, were non-smokers and did not consume alcohol. All donors were informed about the study and gave informed consent to use their blood samples for scientific purposes. Metaphases were collected during the last 2 h with 0.3 μg/ml colchicine. Air-dried preparations were stained by the fluorescense-plus-Giemsa procedure (7).

Assessment of the PRI. The preparations were scored for cells in their first, second and subsequent divisions, with criteria previously described (7) and cells that were suitably spread were scored blindly for SCEs and lymphocyte proliferation kinetics. A minimum of 25 second division cells were scored for each culture in order to establish mean SCE values. For PRIs, at least 100 cells were evaluated. The PRI was calculated according to the formula $PRI=(M_1+2M_2+3M_3^+)/N$, where M_1, M_2 and M_3^+ indicate the number of metaphases corresponding to first, second and third or subsequent divisions, respectively, and N is the total number of metaphases scored.

Statistical evaluation. For the statistical evaluation of the experimental data, the χ^2test was performed for the cell kinetic comparisons. For comparing SCE mean values, the Student's *t*-test was used. Data are presented as mean value±standard error of the mean (SEM).

Results

PK11 and PK15, using the LD_{10} dose, caused a significant ($p<0.001$) reduction in the size of primary LLC in mice (Table II and Figure 2). Fourteen days after implantation, the size of the primary tumours implanted on the right-hind leg

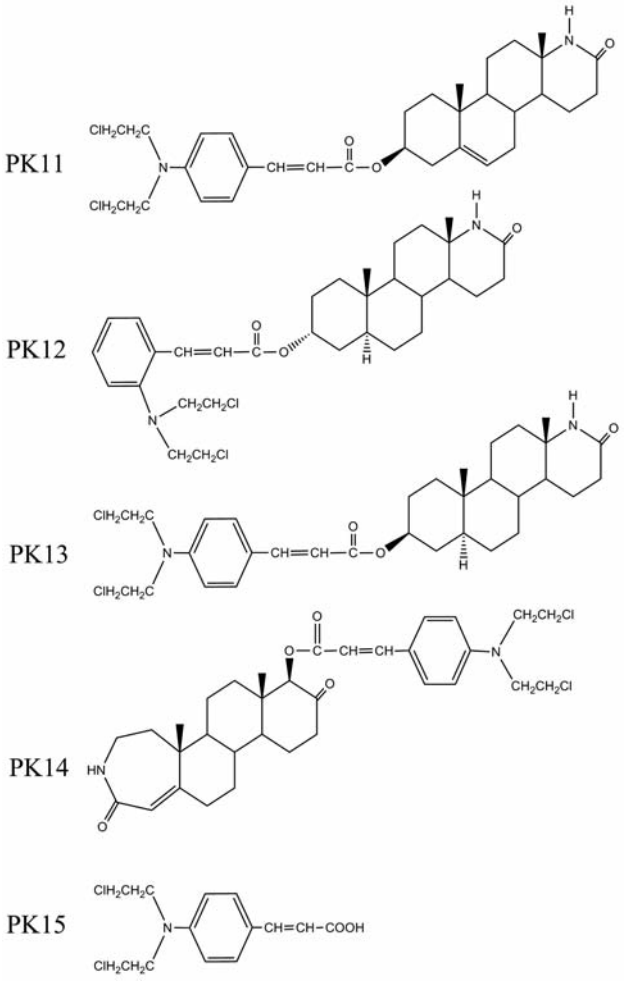

Figure 1. *Chemical structure of modified steroidal derivatives of p-bis(2-chloroethyl)amino phenyl propenoic acid.*

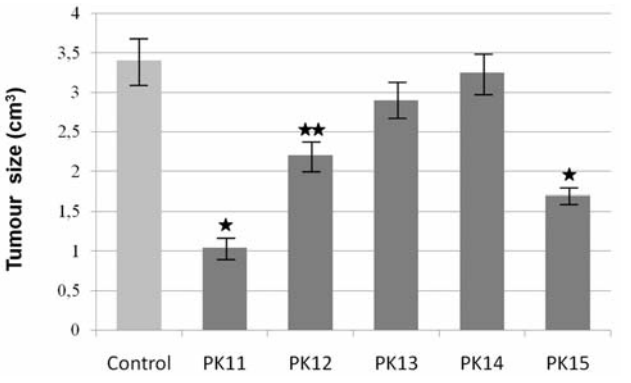

Figure 2. *Antitumour effect of compounds PK11-PK15 on LLC. Results are expressed as mean±SEM (standard error of the mean) and were compared to the controls. Asterisks denote statistical significance between control and compounds tested. *p<0.01, **p>0.05 by unpaired t-test. Eight animals were used in the control group and 6 for each chemical tested.*

Table I. *Acute toxicity of PK11-PK15 in BDF1 female mice.*

Dose (μg/g)	PK11	PK12	PK13	PK14	PK15
800	8/10*	9/10	5/10	10/10	–
600	3/10	5/10	1/10	9/10	–
400	0/10	1/10	0/10	7/10	10/10
200	0/10	0/10	0/10	1/10	9/10
100	0/10	0/10	0/10	0/10	1/10
LD_{50}	700	600	900	350	140
LD_{10}	500	400	600	200	100

*Number of deaths/number of animals in tested group. LD_{10}, LD_{50}: lethal dose for 10%, 50% deaths due toxicity, respectively. Details about the calculation of LD_{10}, LD_{50} are given under Materials and Methods.

Table II. *Antitumour activity of PK11-PK15 against Lewis lung carcinoma (LLC).*

Compounds	Treatment schedule	Dosage (mg/kg)	MTV±SE (cm^3)	ITG (%)
Control	day 1	corn oil	3.40±0.44	0
PK11	day 1	500	1.04±0.13*	69.4
PK12	day 1	400	2.20±0.22**	35.2
PK13	day 1	600	2.90±0.29	14.7
PK14	day 1	200	3.25±0.33	4.4
PK15	day 1	100	1.70±0.08*	50.0

Asterisks denote statistical significance between control and compounds tested: *$p<0.01$, **$p>0.05$ by unpaired *t*-test. MTV: Mean tumour volume; ITG: Inhibition of tumour growth by volume; SE: Standard error.

Table III. *Comparative study on cytogenetic effects induced by modified steroidal derivatives of p-bis(2-chloroethyl)amino phenyl propenoic acid in human lymphocytes.*

Chemical and concentration (μM)		SCEs/cell±SE	Range	PRI
Control I		6.54±0.47	3-11	2.55
PK11	0.2	9.95±0.60	6-15	2.07
	0.6	12.41±0.88	8-18	2.18
PK12	0.2	6.41±0.64	3-10	2.42
	0.6	6.90±0.80	4-11	2.26
PK13	0.2	9.01±0.65	7-13	2.28
	0.6	8.54±0.83	6-14	2.14
PK14	0.2	6.68±0.51	4-10	2.45
	0.6	7.94±0.82	5-16	2.38
PK15	0.2	10.87±0.67	7-14	2.41
	0.6	10.0±1.03	6-18	2.24
Control II		10.34±0.80	6-21	1.92
PK11	2	49.96±2.13	39-64	1.60
	6	69.63±2.86	49-90	1.50
PK12	2	12.80±1.01	7-22	2.04
	6	15.51±1.0	8-23	1.34
PK13	2	15.57±0.97	7-28	1.66
	6	29.27±1.72	18-43	1.58
PK14	2	10.0±0.86	7-17	1.66
	6	9.9±1.19	6-25	1.40
PK15	2	48.3±2.33	45-61	1.51
	6	69.49±2.73	52-86	1.13

A minimum of 25 cells was scored for SCEs from each culture. PRIs were based on at least 100 cells. The PRIs were correlated with the corresponding SCE values (r=–0.68, $p<0.001$). SCE: Sister chromatid exchange; PRI: Proliferation rate index; SE: Standard error.

was 3.4±0.44 cm^3 for the control group, compared to 1.04±0.13 cm^3 for the group which received the LD_{10} dose of PK11 and to 1.7±0.08 cm^3 for the group which received the LD_{10} dose of PK15 (Table II and Figure 2). PK12 and PK13 at the LD_{10} dose caused a non-significant ($p>0.05$) reduction in the size (2.2±0.22 cm^3 and 2.9±0.29 cm^3, respectively for PK12 and PK13) of primary LLC in mice, while PK14 at the LD_{10} dose had no effect at all (3.25±0.33 cm^3) (Table II and Figure II). PK11 and PK15 induced a statistically significant increase in SCE rates at all concentrations tested ($p<0.01$). PK11 and PK15 appeared to be equally effective in inducing SCEs (Table III). PK13 induced a significant increase in SCE levels ($p<0.05$ for the two lower concentrations and $p<0.01$ for the higher concentrations). PK12 had a moderate effect ($p<0.05$) on SCE levels at 2 μM and a more pronounced effect at 6 μM ($p<0.01$). PK11 and PK15 had the strongest effect in suppressing the PRI ($p<0.01$). The results in Table III demonstrated a correlation between the magnitude of the SCE response and the depression of PRI (r=–0.68, $p<0.001$).

Discussion

Due to the delay in the rate of cell turnover, chemically induced cytotoxicity is clearly manifested as a change in the relative proportions of cells in their first, second and subsequent divisions. Studies on the relationship between SCE induction and other expressions of genotoxicity have shown a positive relationship between SCEs and reduced cell survival and alteration in cell cycle kinetics (4).

It is possible that the SCE assay has a predictive value as a clinical assay for drugs in cases where a strong correlation between cell death and induction of SCEs has been established (4, 8, 9). In this study, a sound correlation between SCE induction and cell division delay ($p<0.001$) in the group of five compounds was observed (Table III).

The first four compounds in Figure 1 contain a modified steroid as a biological platform for transporting the alkylating agent (PK15) to the tumour site. Although PK12 and PK13 are isomers, they are slight SCE inducers compared to PK11 and PK15. In PK11 the homoazasteroidal

nucleus has a double bond, while in PK13 it has a single bond. In our experiments, PK11 induced cytogenetic and antineoplastic effects more effectively compared with PK13 (Tables II and III). PK14 which was identified as ineffective in inducing cytogenetic and antineoplastic effects, differs from PK11, PK12 and PK13 in terms of its homoazasteroidal nucleus (Tables II and III and Figure 1). The differential cytogenetic and antineoplastic effects induced by the compounds tested may be due to the previously described differing chemical structures of these chemicals.

An attempt was made to correlate the differential antitumour activity of these compounds (Table II and Figure 2) with their effectiveness in SCE induction (Table III). Other studies have indicated that the effectiveness of antitumour alkylating agents in SCE induction in cancer rodent cells *in vitro* and *in vivo* (8, 9), can be positively correlated with an *in vivo* tumour response to these agents. This suggests that the SCE assay could be used to predict both the sensitivity of human tumour cells to chemotherapeutic agents and the heterogeneity of drug sensitivity within individual tumours (10, 11).

The order of the antitumour effectiveness in LLC of the five compounds tested resembled the order of the cytogenetic damage induced by the same compounds in human lymphocytes *in vitro* (Tables II, III and Figure 2).

The ability to excise and repair various types of damage to DNA is probably a general property of living cells. This ability is of considerable interest to cancer because it provides a mechanism for the modification of the rate of potential genetic damage caused by chemotherapeutics (4). SCEs have been increasingly used as highly sensitive indicators of DNA damage and/or subsequent repair (2, 4). In this context it may be useful to note that tumour cells, like normal human cells, have similar DNA repair mechanisms. Therefore, it is expected that the DNA repair mechanisms in both cell types would be deranged in a similar manner by the same potential chemotherapeutics.

In conclusion, the correlation between SCE induction, suppression of proliferation rate and antitumour activity observed in this study, appeared to further substantiate the validity of the SCE assay as a possible method for improving and guiding chemotherapy (2, 4, 11).

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Received 17 June 2010
Revised 16 July 2010
Accepted 21 July 2010